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Kindlin-3 Mediates Integrin αLβ2 Outside-in Signaling, and It Interacts with Scaffold Protein Receptor for Activated-C Kinase 1 (RACK1)*[S]

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Background: Kindlin-3 is a cytoplasmic protein that binds and modulates the ligand binding property of integrin αLβ2.
Results: Kindlin-3 induces integrin αLβ2 clustering, and it interacts with the scaffold protein RACK1.
Conclusion: Kindlin-3 is involved in integrin αLβ2 outside-in signaling.
Significance: This study presents important findings in understanding the role of kindlin-3 in integrin signaling.

Integrins are heterodimeric type I membrane cell adhesion molecules that are involved in many biological processes. Integrins are bidirectional signal transducers because their cytoplasmic tails are docking sites for cytoskeletal and signaling molecules. Kindlins are cytoplasmic molecules that mediate inside-out signaling and activation of the integrins. The three kindlin paralogs in humans are kindlin-1, -2, and -3. Each of these contains a 4.1-ezrin-radixin-moesin (FERM) domain and a pleckstrin homology domain. Kindlin-3 is expressed in platelets, hematopoietic cells, and endothelial cells. Here we show that kindlin-3 is involved in integrin αLβ2 outside-in signaling. It also promotes micro-clustering of integrin αLβ2. We provide evidence that kindlin-3 interacts with the receptor for activated-C kinase 1 (RACK1), a scaffold protein that folds into a seven-blade propeller. This interaction involves the pleckstrin homology domain of kindlin-3 and blades 5–7 of RACK1. Using the SKW3 human T lymphoma cells, we show that integrin αLβ2 engagement by its ligand ICAM-1 promotes the association of kindlin-3 with RACK1. We also show that kindlin-3 co-localizes with RACK1 in polarized SKW3 cells and human T lymphoblasts. Our findings suggest that kindlin-3 plays an important role in integrin αLβ2 outside-in signaling.

Integrins are a large family of cell adhesion molecules that have important functions in immunity, wound healing, hemostasis, and the development of metazoans (1). Besides mediating cell-cell or/and cell-extracellular matrix interactions, intracellular signals that are derived from integrins regulate cell proliferation, apoptosis, and differentiation (2). In humans there are 24 integrins, and each of these is a heterodimer formed by an α subunit and a β subunit that are non-covalently associated (1). Each subunit contains a large ectodomain and a transmembrane domain followed by a short cytoplasmic tail. Integrins are not enzymes, but they are transducers of bi-directional signals (1). Inside-out signaling involves intracellular signaling events triggered by cell surface receptors, for example chemokine receptor, that regulate the ligand binding affinity of the integrins (3, 4), whereas outside-in signaling involves signaling pathways derived from ligand-bound integrins and/or integrin clustering.

The integrin cytoplasmic tails mediate both inside-out and outside-in signaling events. The integrin tails are docking sites for cytoskeletal and signaling proteins (4, 5). The integrin β tails contain two highly conserved N*XX*(Y/F) motifs. Talins (1 and 2) are large cytoskeletal proteins, each containing a 4.1-ezrin-radixin-moesin (FERM)[2]-containing head domain (HD) and a helical rod region (6). The talin FERM domain is composed of F1, F2, and F3 subdomains, and the latter contains a phosphotyrosine binding fold that allows talin to bind to the membrane proximal N*XX*(Y/F) motif in the integrin β tails, leading to the separation of the integrin α and β tails (7–10). This triggers further structural changes in the integrin that involves unpacking the transmembrane domains and unfolding of the ectodomain, leading to an increase in the ligand binding affinity of the integrin (11, 12).

Kindlins (1, 2, and 3) are another family of FERM-containing cytoplasmic proteins that have been reported to regulate integrin ligand binding affinity (13–15). The F3 subdomain of kindlins binds to the membrane-distal N*XX*(Y/F) motif in the integrin β tails (13, 15, 16). Each kindlin also contains an F0 and a pleckstrin homology (PH) domain (17, 18). The F0 domain has an ubiquitin-like fold that targets kindlin-1 to integrin αIIbβ3-containing focal adhesion sites (18). The PH domain has been shown to facilitate kindlin-2 binding to membrane phosphatidylinositol 3,4,5-trisphosphate (19). The detailed mechanism by which kindlin regulates the ligand binding affinity of integrins remains to be clarified. Besides integrins, the cytoplasmic

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[2] The abbreviations used are: FERM, 4.1-ezrin-radixin-moesin; PH, pleckstrin homology; RACK1, receptor for activated-C kinase 1; CFP, cyan fluorescent protein; mCFP, monomeric CFP; YFP, yellow fluorescent protein; mYFP, monomeric YFP; RT, room temperature; ICAM, intercellular adhesion molecule.

molecules integrin-linked kinase and migfilin have been reported to interact with kindlin-2 (20, 21). Migfilin has been shown to be a positive regulator of integrin activation because it disrupts the binding of cytoskeletal protein filamin A to the integrin β tail (22, 23). Filamin A is a negative regulator of integrin activation because it competes with talin for binding the integrin β tail (24). Hence, kindlin-migfilin interaction promotes integrin activation.

Kindlin-3 (UNC112-related protein 2 or FERMT3) is expressed in platelets, hematopoietic cells, and endothelial cells (25, 26). Its importance is underscored by the disease leukocyte adhesion deficiency III in which defective kindlin-3 expression abrogated the functions of platelet αIIbβ3 and leukocyte αLβ2 integrins, leading to bleeding disorders and a compromised immune system in patients, respectively (27–30). Mice with ablated *KINDLIN-3* phenocopied leukocyte adhesion deficiency III but also had severe osteopetrosis and structural defects in the membranes of their erythrocytes (14, 15, 31). *KINDLIN-3*$^{-/-}$ platelets adhered but failed to spread on immobilized fibrinogen despite activation of integrin αIIbβ3 by exogenous $MnCl_2$ which bypassed inside-out signaling (15). This suggests that kindlin-3 is also required for integrin outside-in signaling, and it may interact with cytoplasmic protein(s) that modulate cell spreading.

Here we show that kindlin-3 is involved in integrin αLβ2 outside-in signaling, and it induces integrin αLβ2 micro-clustering. We also show that kindlin-3 can interact with the receptor for activated-C kinase 1 (RACK1), a small (36 kDa) seven-blade propeller WD-repeat scaffold protein with multiple cytosolic binding partners (32), including the integrin β cytoplasmic tails (33–35). Using the human T lymphoma cell line SKW3, we show that integrin αLβ2 engagement by its ligand ICAM-1 promotes kindlin-3 and RACK1 interaction. Finally, we observed that kindlin-3 and RACK1 co-localized in polarized SKW3 cells and human T lymphoblasts that adhered to immobilized ICAM-1.

EXPERIMENTAL PROCEDURES

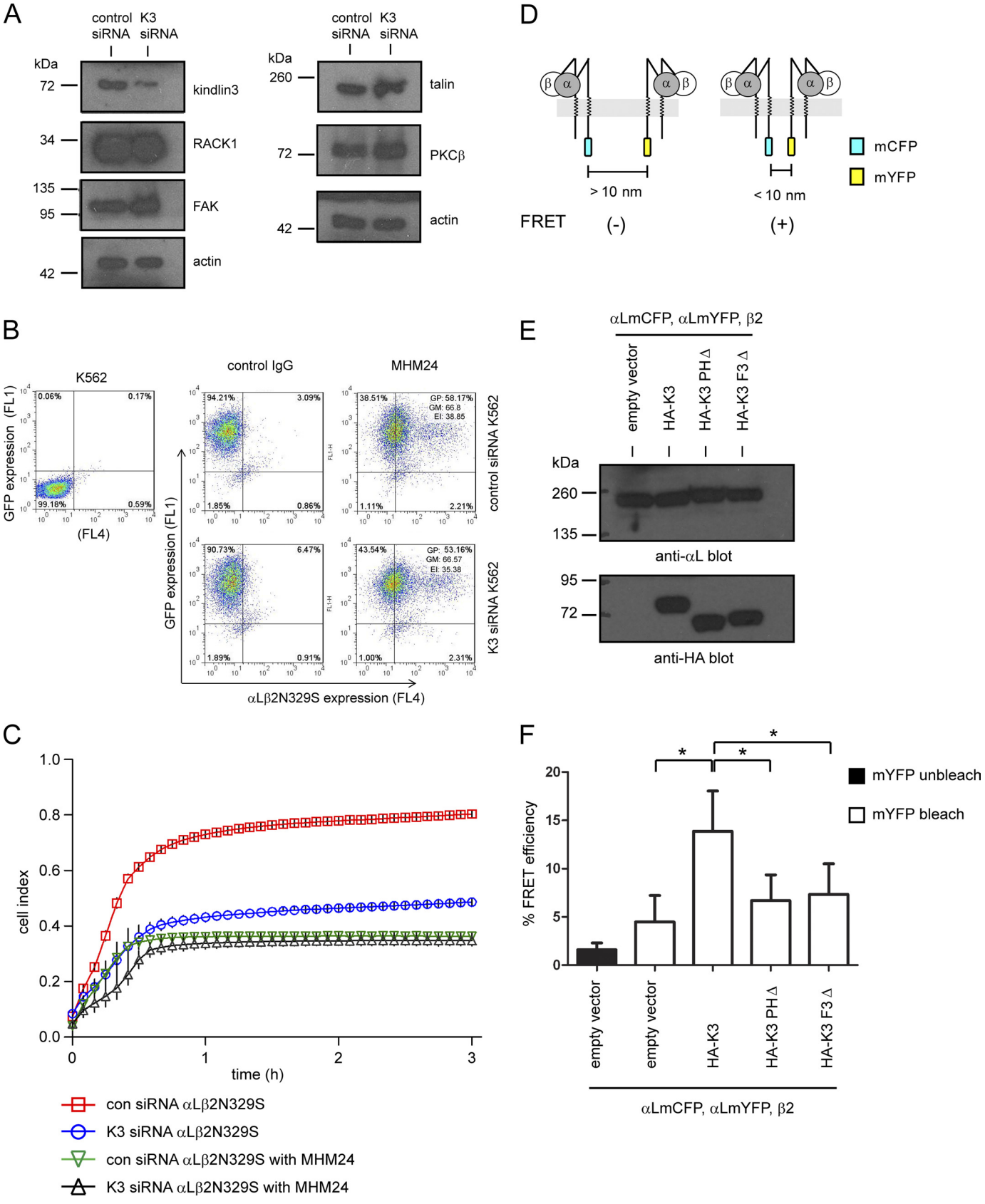
Antibodies—The mAb MHM24 hybridoma (integrin α_L-specific, function-blocking) was a kind gift from Prof. A. J. McMichael (John Radcliffe Hospital, Oxford, UK) (36). The purified mAb 10E5 (integrin αIIb-specific, function-blocking) was kindly provided by Prof. B. S. Coller (The Rockefeller University, New York, NY) (37, 38). The following antibodies were purchased from different sources. Mouse anti-RACK1 mAb (clone B-3) and rabbit anti-MyH9 polyclonal antibody were from Santa Cruz Biotechnology Inc., Santa Cruz, CA. Mouse anti-integrin αL mAb (clone 27), mouse anti-actin mAb (clone C4), and mouse anti-PKCβ were from BD Biosciences. Rabbit anti-HA polyclonal antibody was from Delta Biolabs, Gilroy, CA. Rabbit anti-FAK polyclonal antibody was from Cell Signaling Technology, Danvers, MA. Mouse anti-talin (8d4), rat IgG, and mouse IgG (mAb MOPC-31c) were from Sigma. Rabbit anti-kindlin-2 polyclonal antibody was from ProteinTech Group, Chicago, IL. HRP-conjugated donkey anti-rabbit IgG, HRP-conjugated sheep anti-mouse IgG, and HRP-conjugated goat anti-rat IgG were from GE Healthcare. Mouse anti-kindlin-3 polyclonal antibody was from Abnova, Taipei, Taiwan. We have also generated rat anti-kindlin-3 mAbs clone 9 and clone 229A using recombinant full-length human kindlin-3 as the immunogen.

Generation of Stable K562 Cells with Reduced Kindlin-3 Expression—Stable K562 cells with reduced kindlin-3 expression were generated using a third generation lentiviral-based siRNA transduction system according to the manufacturer's instructions (Applied Biological Materials). Briefly, four kindlin-3-targeting piLenti-RNAi-GFP plasmids were generated. Each of these plasmids or the control siRNA encoding plasmid was co-transfected with the packaging plasmids into 293T cells to allow production of pseudo-virions, and the culture supernatant was collected. K562 cells (1×10^6) were resuspended in 1 ml of complete RPMI 1640 medium supplemented with 6 μg/ml Polybrene (Sigma) followed by the addition of 1 ml of the pseudo-virions-containing supernatant, and the cells were cultured for 1 day. Cells were then spun down and cultured in complete RPMI 1640 medium for at least 2 days. GFP expression in cells was visualized under a fluorescence microscope. Thereafter, cells stably expressing kindlin-3 siRNA were selected in the presence of puromycin (1.5 μg/ml). Only one of the four kindlin-3-targeting piLenti-RNAi-GFP plasmids produced K562 clones that showed significant reduction in kindlin-3 expression. The sequence that encodes this kindlin-3 siRNA is TGGAGCAGATCAATCGCAA, which has been previously described (29).

Flow Cytometry Analyses—Flow cytometry analyses of cells were performed as previously described (39). Integrin transfectants were incubated with 20 μg/ml each of mAb MHM24 (for αLβ2 or αLβ2N329S) or mAb 10E5 (for αIIbβ3 and αIIbβ3N339S) in medium on ice for 30 min. Cells were washed in medium and incubated in medium containing APC-conjugated goat anti-mouse IgG (BD Pharmingen) (1:400 dilution) on ice for 30 min. Sample acquisitions were performed on a FACSCalibur flow cytometer (BD Biosciences). Data were analyzed using the Flowjo software (Tree Star Inc. Ashland, OR).

Surface Plasmon Resonance Analyses—Sensor chip CM5, *N*-hydroxysuccinimide (NHS), *N*-ethyl-*N*-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), ethanolamine, and surfactant P20 were purchased from Biacore, GE Healthcare. Surface plasmon resonance assays were run at 30 μl/min at 37 °C on a Biacore 3000 system. Standard HBS-EP buffer (150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P20, 50 mM HEPES, pH 8.0) was used for the analysis. Sensorgrams produced from Tris buffer used in protein purification were similar to those from HBS-EP buffer. GST-RACK1 was immobilized to 5000 response units onto sensor chip CM5 using a standard amine-coupling procedure as previously described (40). GST was also immobilized to the same level onto another flow cell as control. Kindlin-3-His_6 was verified not to interact with immobilized control GST. 2-Fold serially diluted kindlin-3-His_6 (1.56, 3.125, 6.25, 12.5, 25, 50 and 100 μM) were separately injected across flow cells comprising the control GST surface or GST-RACK1 for 6 min, allowed to dissociate for 5 min, and regenerated with a short pulse (30 s) of 15 mM HCl. All raw sensorgrams were double-referenced (41) by subtracting all resultant sensorgrams with the con-

trol GST surface (to eliminate nonspecific binding) and blank buffer injections (to eliminate equipment systematic error) and then globally fit to a simple bimolecular interaction model. Three replicates of the interaction were performed (with different batches of proteins) with similar results.



Pulldown Assays—GST, GST-RACK1, and GST-RACK1 W5-7 were affinity-purified on glutathione S-Sepharose® 4B columns. The GST protein-bound beads were incubated in buffer A (150 mM NaCl, 10% (v/v) glycerol, 50 mM Tris, pH 8.0) containing 5% (w/v) BSA at 4 °C for 1 h to block nonspecific binding sites. The beads were subjected to four washes in buffer A before use. Different amounts of purified kindlin-3 or its mutants were incubated with these beads in buffer A at 4 °C overnight. Beads were subjected to four washes in buffer A, and bound proteins were eluted by boiling the beads in SDS-PAGE sample buffer containing DTT.

The N-terminal biotin-conjugated full-length integrin β2 tail peptide (Lys-702–Ser-747) and its mutant (N741A) were synthesized, purified, and ESI-MS-verified by ChinaPeptides, Shanghai, China. The peptide (100 μg each) was incubated with 50 μl of streptavidin-agarose beads suspension (Sigma) in buffer C (150 mM NaCl and 50 mM Tris, pH 8.0) at 4 °C for 1 h. The beads were washed and incubated in the same buffer containing 0.5% (w/v) BSA at 4 °C for 1 h to block nonspecific binding sites. Thereafter, washed beads were incubated with indicated amount of purified recombinant kindlin-3-His_6 or His_6-RACK1.

FRET Analyses—COS-7 transfectants expressing CFP and YFP-RACK1, CFP-kindlin-3 and YFP-RACK1, CFP-kindlin-3 PHΔ and YFP-RACK1, or CFP-kindlin-3 F3Δ and YFP-RACK1 were seeded into coverslip glass-bottom culture dishes and cultured for 24 h. FRET analyses were performed as previously described (42, 43) on a Zeiss LSM510 confocal laser scanning microscope (Carl Zeiss, Inc., Thornwood, NY) to detect the interaction between RACK1 and kindlin-3 or mutants. The nucleus of the cell was excluded from the analyses. FRET efficiency was calculated as a percentage using the equation FRET efficiency = $(I_6 - I_5) \times 100/I_6$, where I_n is the CFP intensity at the nth time point. Bleaching of YFP was performed between the fifth and sixth time points. For FRET analyses of integrin αLβ2 clustering, K562 cells were transfected with HA-tagged kindlin-3 or its mutants with αLmCFP, αLmYFP, and β2. Only the plasma membrane was selected as the region of interest for measurements of CFP signal before and after YFP photobleaching.

Real-time Electric Cell-Substrate Impedance Sensing Measurements—Dithiobis succinimidyl propionate (Pierce, Thermo Fisher Scientific, Rockford, IL) (4 mg/ml) in DMSO (40 μl) was added to each well of a 16-well E-plate® device (Acea Biosciences, Inc., San Diego, CA) with gold electrodes at the bottom of each well and incubated for 30 min at RT. Wells were washed twice in deionized H_2O. For the integrin αLβ2N329S assay, goat anti-human Fc (Sigma) (10 μg/ml) in PBS was added to each well and incubated for 1 h at RT. Nonspecific binding sites were blocked with 0.1% (w/v) BSA in PBS for 15 min at RT. Wells were coated with 2 μg/ml ICAM-1 Fc in PBS (50 μl per well) and incubated for 2 h at RT. For the integrin αIIbβ3N339S assay, 1 μg/ml fibrinogen (Sigma) in PBS was added to each well and incubated for 1 h at RT followed by blocking with 0.1% (w/v) BSA in PBS for 15 min at RT. Wells were washed once in RPMI 1640 complete medium, and each well was refilled with 100 μl of medium. Background scans of the wells were performed on a Real Time Cell Electronic System™ (Acea Biosciences, Inc.). K562 transfectants (8×10^4 cells per well) were seeded into each well, and AC impedance (cell index) measurements taken at 1-min intervals. Cell adhesion and spreading mediated by integrins αLβ2N329S and αIIbβ3N339S were verified by having function-blocking mAbs MHM24 and 10E5 (10 μg/ml each), respectively. For clarity, cell index is plotted as a function of time at 5-min intervals.

Immunofluorescence Staining and Imaging—A coverslip glass-bottom tissue culture dish (MatTek, Ashland, MA) was coated with goat anti-human IgG Fc (5 μg/ml) in bicarbonate buffer at 37 °C for 1 h. Nonspecific binding sites were blocked with 0.5% (w/v) BSA in PBS at 37 °C for 30 min. The culture dish was then coated with ICAM-1-Fc (1 μg/ml) in PBS at 4 °C overnight. T cells were resuspended in RPMI complete medium containing SDF-1α (100 ng/ml) (EMD4 Biosciences, Gibbstown, NJ), seeded into the dish, and incubated in a cell culture incubator for 15 min. Medium was discarded, and the cells were fixed in 3.7% (w/v) paraformaldehyde in PBS at RT for 10 min. Fixed cells were washed once in cytoskeleton stabilization buffer (100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 10 mM PIPES, pH 6.8) containing 0.3% (v/v) Triton X-100 followed by incubation in the same buffer at RT for 1 min. Cells were subjected to 3 washes in PBS, and nonspecific sites were blocked with 3% (w/v) BSA in PBS at RT for 30 min. Cells were incubated in PBS containing relevant primary antibodies at RT for 1 h. The primary antibodies used were mouse anti-RACK1 mAb (clone B-3), rat anti-kindlin-3 mAb (clone 9), and rabbit anti-MyH9 polyclonal antibody. Cells were then washed in PBS. For co-staining with actin, cells were incubated in PBS containing highly cross-adsorbed Alexa Fluor® 594-conjugated goat anti-mouse IgG, donkey anti-rat IgG, or goat anti-rabbit IgG antibodies (1:10,000 dilution each) (Molecular

FIGURE 1. **Kindlin-3 mediates integrin αLβ2 outside-in signaling and promotes integrin αLβ2 micro-clustering.** *A*, cell lysates from K562 stably expressing control siRNA or kindlin-3 (*K3*)-targeting siRNA were subjected to immunoblotting to assess the expression levels of kindlin-3 and other cytoplasmic proteins. Actin was used as loading control. *B*, K562 cells were successfully and stably transduced with the lentiviral-based siRNA with GFP as the reporter was transfected with integrin mutant αLβ2N329S. Expression level of integrin αLβ2N329S was determined by staining transfectants with mAb MHM24 followed by APC-conjugated secondary antibody. Two-color flow cytometry analyses were performed. *FL1* was for GFP expression, and *FL4* was for integrin αLβ2N329S expression. Expression index (*EI*) was calculated by the percentage of cells gated positive (*GP*) × geo-mean fluorescence intensity (*GM*). *C*, shown is electric cell-substrate impedance sensing measurements of these K562 transfectants on immobilized ICAM-1. The function-blocking mAb MHM24 was included to demonstrate integrin αLβ2-mediated binding specificity. Each data point is the mean ± S.D. (*gray bar*) of technical triplicates. A representative plot of three independent experiments is shown. Measurements were taken at 1-min intervals, but for clarity cell index is plotted as a function of time at 5-min intervals. *D*, the drawing illustrates the principle of FRET-based detection of integrin αLβ2 micro-clustering using the αL subunit having a C-terminal fusion of mCFP or mYFP as previously reported (9, 43). *E*, shown are immunoblots of cell lysates of K562 transfected with integrin subunits αLmCFP, αLmYFP, β2, and HA-tagged kindlin-3 constructs. *F*, shown are FRET analyses of integrin αLβ2 micro-clustering in K562 transfectants. Each data point represents the mean ± S.D. of ≥40 cells analyzed. *, $p < 0.05$, Student's *t* test. A representative plot of three independent experiments is shown.

Probes, Invitrogen) with Alexa Fluor® 488-conjugated phalloidin (0.27 ng/ml) and DAPI (0.1 μg/ml) at RT for 1 h. Cells were then subjected to 3 washes in PBS. Images were acquired on a Zeiss LSM 510 confocal laser scanning microscope with a 63× oil objective lens. Images were analyzed using the LSM 510 META software Version 3.2 SP2. Thermal LUT surface plots (intensity plots) were generated using the Image J software.

See supplemental Experimental Procedures for expression plasmids, cell culture and transfection, immunoprecipitation assays and immunoblotting, and expression and purification of recombinant proteins from *Escherichia coli*.

RESULTS

Kindlin-3 Is Required for Integrin αLβ2 Outside-in Signaling—Kindlins are co-activators, together with talin, of integrins. Kindlin-3 is well reported to be important for β2 integrin-mediated leukocyte adhesion and spreading (15, 25, 28, 30, 44). However, direct evidence of kindlin-3 in β2 integrin outside-in signaling is lacking. To this end we made use of a lentiviral-based siRNA transduction system with GFP reporter to generate stable K562 cell line that expressed either siRNA-targeting kindlin-3 or control siRNA. Cells transduced with siRNA-targeting kindlin-3 showed reduced kindlin-3 expression compared with cells transduced with control siRNA (Fig. 1*A*). The expression levels of cytoplasmic proteins RACK1, FAK, talin, and PKCβ in siRNA-targeting kindlin-3 and control siRNA cells were comparable. K562 cells do not express endogenous β2 integrins. K562 cells were subsequently transfected with the integrin mutant αLβ2N329S, which has been shown previously to be activated constitutively (45). The rationale to use a constitutively activated mutant is to bypass the inside-out activation process that requires kindlin-3. This allows us to examine the role of kindlin-3 in integrin αLβ2 outside-in signaling in K562 cells with reduced kindlin-3 expression. The expression levels of integrin αLβ2N329S on the transfectants were comparable based on mAb MHM24 staining and flow cytometry analyses (Fig. 1*B*). Both transfectants were also GFP-positive compared with wild-type K562 cells that were not transduced with the lentiviral siRNA.

We performed real-time electrical cell-substrate impedance sensing measurements (46) of these cells on immobilized ICAM-1. Impedance sensing is reported as cell index herein. Cell index increases when cells adhere and spread on the electrode. The cell index of control siRNA cells expressing integrin αLβ2N329S increased markedly over time, whereas that of kindlin-3 siRNA cells expressing integrin αLβ2N329S was marginal (Fig. 1*C*). The specificity of integrin αLβ2N329S-mediated adhesion and spreading was demonstrated by including the function-blocking mAb MHM24 (anti-αL). These data suggest that kindlin-3 is involved in integrin αLβ2 outside-in signaling as K562 cells, with reduced kindlin-3 expression failed to adhere and spread effectively despite expressing constitutively activated integrin αLβ2. The generality of kindlin-3 in mediating integrin outside-in signaling was also shown using cells that expressed constitutively activated integrin αIIbβ3N339S (45) (supplemental Fig. S1).

Overexpression of Kindlin-3 in K562 Cells Induced Integrin αLβ2 Micro-clustering—It is not known if kindlin-3 promotes micro-clustering of integrins. We made use of a YFP-photobleach FRET-based assay (43) to determine whether kindlin-3 promotes integrin αLβ2 micro-clustering on K562 cells. αLmCFP and αLmYFP are fusion proteins of integrin αL subunit with C-terminal monomeric CFP and monomeric YFP, respectively (9). When micro-clustering of integrin αLβ2 occurs and if an αLmCFP is in the proximity (<10 nm) of an αLmYFP, FRET should be detected (Fig. 1*D*). Conversely, FRET would be minimal if the molecules are >10 nm apart. K562 cells were co-transfected with four plasmids: αLmCFP, αLmYFP, β2 with empty vector or HA-kindlin-3 (wild-type full-length) or HA-kindlin-3PHΔ (PH domain deleted) or HA-kindlin-3F3Δ (F3 domain deleted). The expression levels of integrin αLmCFP/mYFP and kindlin-3 constructs were verified by immunoblottings (Fig. 1*E*). Significant level of FRET was detected in cells ectopically expressing HA-kindlin-3 compared with cells transfected with empty vector or HA-kindlin-3PHΔ or HA-kindlin-3F3Δ (Fig. 1*F*).

These data suggest that kindlin-3 could induce integrin αLβ2 micro-clustering and both its PH and F3 domains are required for this function. The F3 domain is required for kindlin-3 binding to the integrin β tail, accounting for the lack of integrin αLβ2 micro-clustering in cells overexpressing HA-kindlin-3F3Δ (13, 15, 16). Although it remains to be shown that kindlin-3 PH domain has similar phosphatidylinositol phosphate(s) binding properties to kindlin-2 (19), it is possible that the PH domain allows kindlin-3 to tether at membrane microdomains enriched in phosphatidylinositol phosphate(s), thereby promoting integrin αLβ2 micro-clustering.

Kindlin-3 Interacts with Scaffold Protein RACK1—The seven-blade propeller WD-repeat scaffold protein RACK1 has been shown to bind integrin β tails, including the integrin β2 tail (33, 35). RACK1 blades 5–7 apparently binds the membrane proximal sequence Lys-702–His-706 of the integrin β2 tail, and it co-immunoprecipitated with integrin αLβ2 from lysate of phorbol 12-myristate 13-acetate-treated JY cells (33). It has been reported that RACK1 localized at the site in Jurkat T cell that was in contact with chemokine SDF-1-coated latex bead and at the migratory front of formylmethionylleucylphenylalanine-treated HL-60 cells (47). RACK1 has also been shown to bind PH domains (48). These data prompted us to examine the association of kindlin-3 with RACK1.

The co-immunoprecipitation assay was performed to examine the interaction of endogenous RACK1 with ectopically expressed HA-kindlin-3 (wild type and full-length) or its mutants or HA-kindlin-2 (wild type and full-length) in 293T cells (Fig. 2*A*). Kindlin-2 was included in the assay because it has been reported to be important in cell adhesion and spreading in many biological processes, including angiogenesis, cancer, and wound healing (49–51). RACK1 co-precipitated with HA-kindlin-3 and HA-kindlin-3 F3Δ but not HA-kindlin-2 and HA-kindlin-3 PHΔ. The lack of interaction between kindlin-2 and RACK1 prompted us to perform pulldown assays using recombinant purified GST-RACK1, kindlin-2-His_6, and kindlin-3-His_6 (supplemental Fig. S2). The results from the pull-

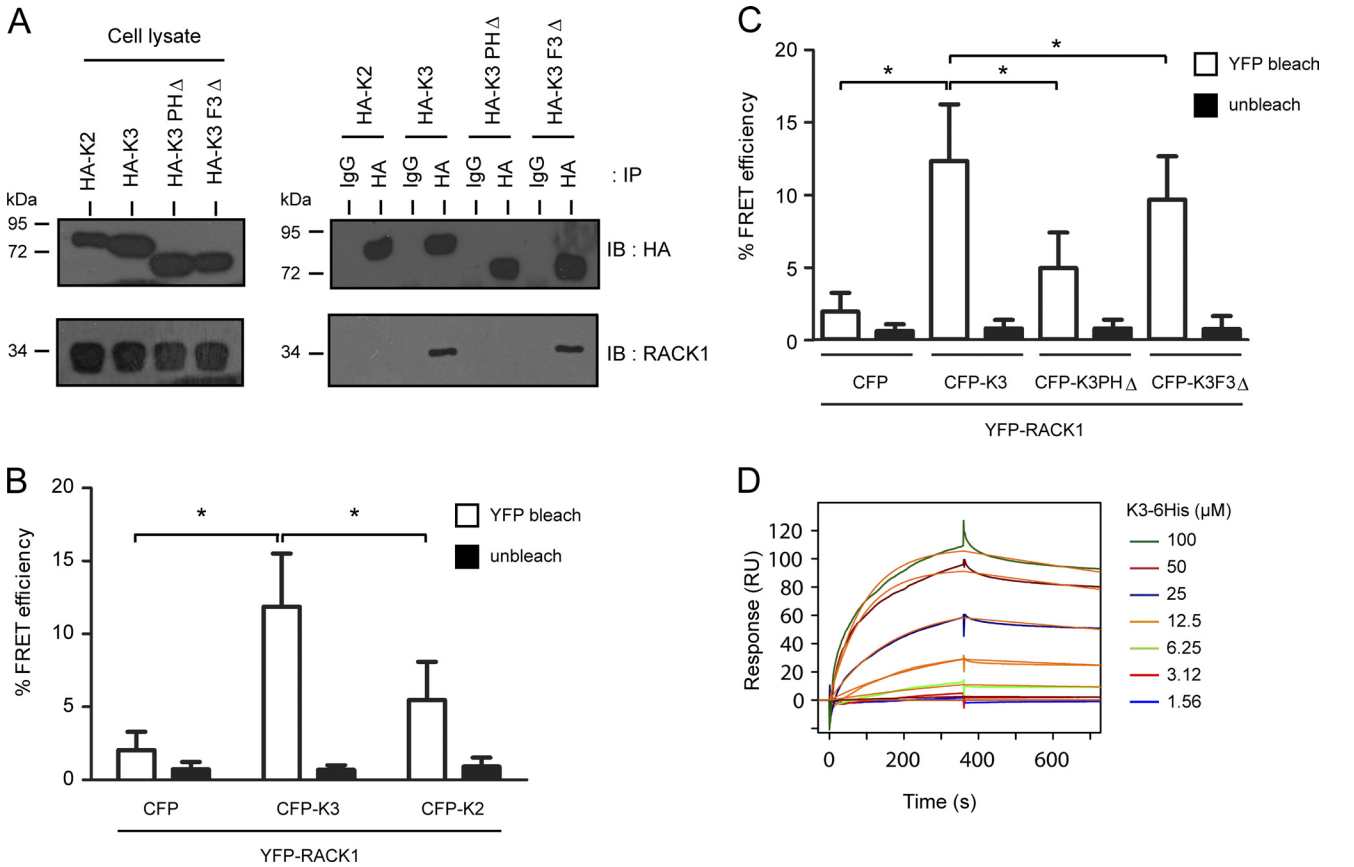


FIGURE 2. **Kindlin-3 interacts with the scaffold protein RACK1.** *A*, 293T cells transfected with HA-tagged kindlin-2 (*K2*), HA-tagged kindlin-3 (*K3*), or its mutants were subjected to immunoprecipitation (*IP*) analyses. Co-precipitated endogenous RACK1 was detected by immunoblotting (*IB*). *B* and *C*, shown are FRET analyses of COS-7 cells transfected with different CFP constructs and YFP-RACK1. Each data point represents the mean ± S.D. of ≥50 cells analyzed. * $p <$ 0.05, Student's *t* test. A representative plot of three independent experiments is shown for each panel. *D*, surface plasmon resonance analyses are shown of K3 and RACK1 interaction. Different concentrations of purified K3-His_6 were injected across the surface of GST or GST-RACK-1-coated flow cells in the CM5 sensor chip. The sensorgrams from GST-RACK1 flow cell were double-referenced (41) by subtracting all resultant sensorgrams with the control GST surface (to eliminate nonspecific binding) and blank buffer injections (to eliminate equipment systematic error). The experimental data (*colored lines*) conformed well to a simple bimolecular model (*orange lines*) with $k_a = 0.2 \times 10^4\ \text{M}^{-1}\text{s}^{-1}$, $k_d = 4.1 \times 10^{-4}\ \text{s}^{-1}$, $K_D = 1.8\ \mu\text{M}$. Three replicates of different batches of proteins gave an average affinity constant of 1.60 ± 0.08 μM. *RU*, response units.

down assays are consistent with the co-immunoprecipitation data.

Next, we performed FRET-based interaction studies in cells. COS-7 cells were transfected with YFP-RACK1 and CFP-kindlin-3, CFP-kindlin-2, or CFP empty vector followed by YFP-photobleach FRET analyses (Fig. 2*B*). A significant level of FRET was detected in cells expressing YFP-RACK1 and CFP-kindlin-3 compared with others. COS-7 cells were also transfected with YFP-RACK1 and CFP-kindlin-3 or its mutants or CFP empty vector followed by FRET analyses (Fig. 2*C*). The levels of FRET detected are on the order of CFP-kindlin-3 > CFP-kindlin-3 F3Δ > CFP-kindlin-3 PHΔ > CFP. These observations suggest that the PH domain and to a lesser extent the F3 domain of kindlin-3 is involved in RACK1 interaction.

To obtain binding constants for the interaction between kindlin-3 and RACK1, we performed surface plasmon resonance by immobilizing GST-RACK1 on a CM5 sensor chip followed by injections of kindlin-3-His_6 at different concentrations (Fig. 2*D*). The experimental data (colored lines) conformed well to a simple bimolecular model (*orange lines*) with $k_a = 0.2 \times 10^4\ \text{M}^{-1}\text{s}^{-1}$, $k_D = 4.1 \times 10^{-4}\ \text{s}^{-1}$, $K_D = 1.8\ \mu\text{M}$. Three replicates using different batches of proteins gave an average affinity constant of 1.60 ± 0.08 μM.

In the next series of experiments, GST pulldown assays using recombinant purified proteins were performed to determine the interaction sites of kindlin-3 and RACK1. Consistent with the previous data, kindlin-3-His_6 and kindlin-3 F3Δ-His_6, but not kindlin-3 PHΔ-His_6, interacted with GST-RACK1 (Fig. 3*A*). No interaction between the kindlin-3 proteins with control GST was detected. RACK1 folds into a propeller containing seven blades, which are docking sites for different cytoplasmic proteins (32). To examine which of these blades of RACK1 interacts with kindlin-3, we attempted to express and purify these blades with an N-terminal GST tag. However, in our hands only GST-RACK1 blades 5–7 Gly-190–Arg-317 could be expressed in soluble form (data not shown). Kindlin-3-His_6 was detected in pulldown samples of GST-RACK1 and GST-RACK1 W5-7 but not control GST (Fig. 3*B*).

We also attempted to express kindlin-3 PH domain to test its association with RACK1 W5-7. The yield of kindlin-3 PH domain in *E. coli* expression system was low, and it was prone to degradation. To circumvent this problem, we constructed and were able to express kindlin-3 PH domain with its flanking F2 subdomains (His_6-kindlin-3 PH-F2), which associated with GST-RACK1 or GST-RACK1 W5-7 but not control GST (Fig. 3*C*). Taken together, these data suggest

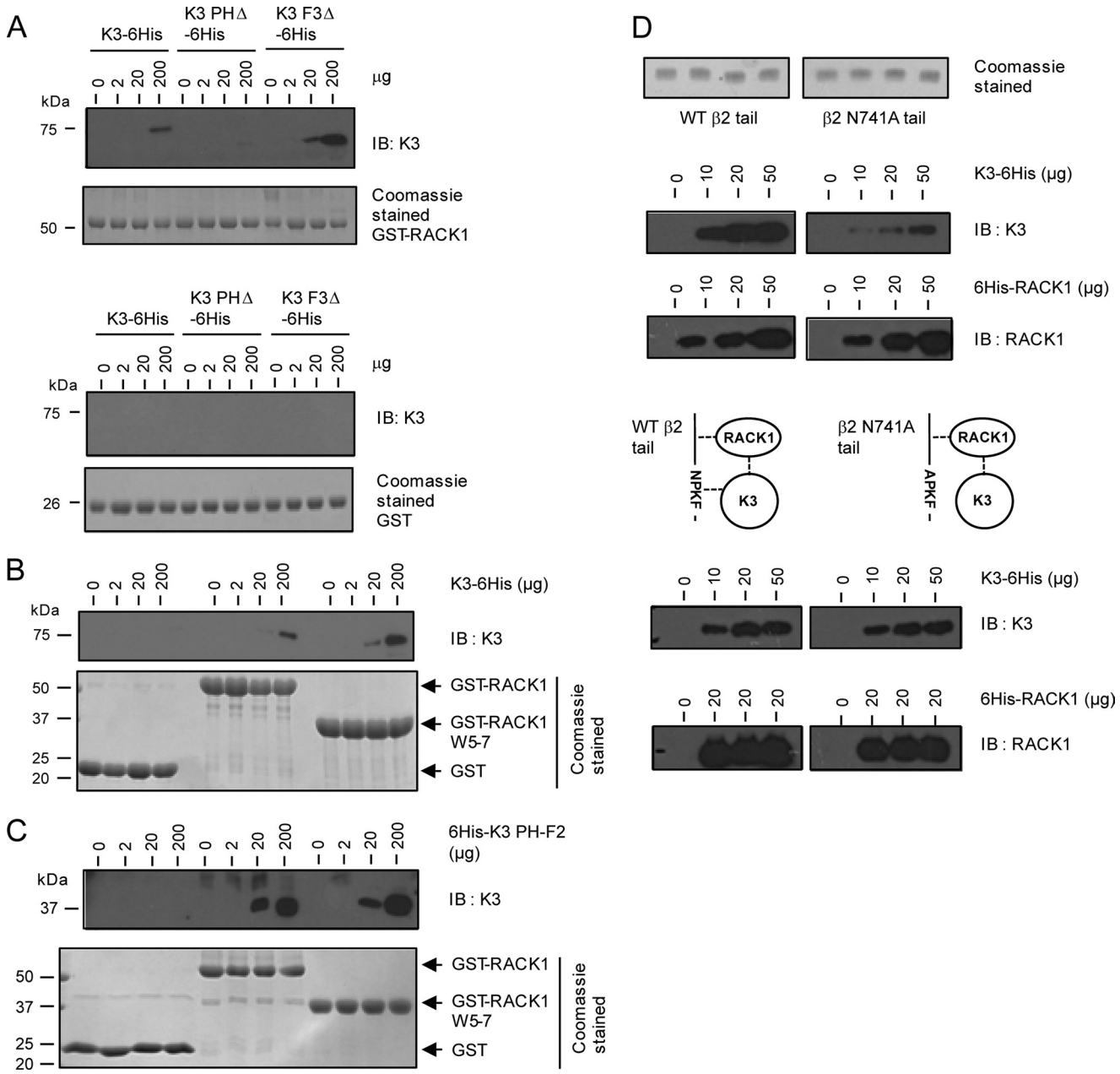


FIGURE 3. **Pulldown assays using recombinant kindlin-3 (*K3*) and RACK1 proteins.** *A*, *B*, and *C*, shown are GST pulldown assays using purified recombinant proteins of K3 and RACK1 and their mutants. Immunoblotting (*IB*) of K3 was performed using commercial anti-kindlin-3 antibody. *D*, shown are pulldown assays using N-terminal biotin labeled wild-type β2 tail or β2 N741A integrin tail-peptide bound to streptavidin-agarose beads and purified recombinant K3-His_6 and His_6-RACK1. Coomassie-stained gels of wild-type β2 or β2 N741A integrin tail peptide bound to streptavidin-agarose beads are shown. *Top panel*, shown are immunoblots of K3-His_6 or His_6-RACK1 associated with wild-type β2 or β2 N741A integrin tail-peptide. *Middle panel*, shown is an illustration of ternary complex formation of integrin β2 tail, kindlin-3, and RACK1. Because kindlin-3 interacts with RACK1, a ternary complex of integrin β2 N741A, kindlin-3, and RACK1 could be formed. The *dotted line* denotes interaction. *Bottom panel*, shown are immunoblots of K3-His_6 associated with wild-type β2 or β2 N741A integrin tail-peptide in the presence of His_6-RACK1.

that blades 5–7 of RACK1 interact with the PH-F2 domain of kindlin-3.

RACK1 has been reported to associate with the integrin β2 tail (34). We asked if the integrin β2 tail, RACK1, and kindlin-3 can form a ternary complex. To this end, N-terminal biotin-labeled full-length wild-type β2 or mutant β2 N741A integrin tails were conjugated to streptavidin-agarose beads. Kindlins have been reported to bind to the membrane distal N*XX*(F/Y) motif of the integrin β tails (13, 52); hence, we included the β2 N741A mutant in which Asn of the membrane distal ^{741}NPKF motif in the β2 tail was replaced with Ala. Pulldown assays were performed using these integrin β2 tail-peptide-bound beads and different amounts of purified recombinant full-length kindlin-3-His_6 or His_6-RACK1 (Fig. 3*D*, *top panel*). The association of kindlin-3-His_6 with wild-type β2 tail was concentration-dependent. By contrast, the level of kindlin-3-His_6 that associated with β2 N741A was diminished. The levels of His_6-RACK1 with wild-type β2 tail and His_6-RACK1 with N741A β2 tail were comparable, suggesting that RACK1 interaction with integrin β2 tail is not dependent on the membrane distal

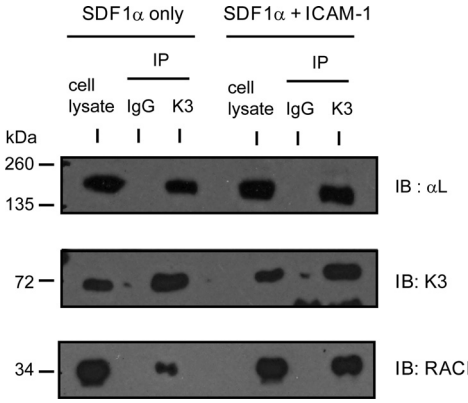


FIGURE 4. **Co-immunoprecipitation of endogenous kindlin-3 with RACK1.** SKW3 human T lymphoma cells were seeded into empty or ICAM-1-coated microtiter wells in the presence of SDF-1α. After incubation for 30 min, cells were harvested, and immunoprecipitation (*IP*) was performed to assess the interaction between kindlin-3 and RACK1. Endogenous kindlin-3 was immunoprecipitated with anti-kindlin-3 mAb (clone 9). An immunoblot (*IB*) of precipitated kindlin-3 was performed using anti-kindlin-3 (clone 229A). Immunoblots of co-precipitated endogenous RACK1 and integrin αLβ2 were performed using anti-RACK1 mAb (clone B-3) and anti-αL mAb (clone 27), respectively. *IgG*, control rat IgG.

^{741}NPKF motif. This is in line with the observation that RACK1 interacts with the membrane proximal sequence of the integrin β2 tail (33).

Next we asked whether integrin β2 tail, kindlin-3, and RACK1 can form a ternary complex. We incubated the integrin β2 tail-peptide-conjugated beads with the same amount of His_6-RACK1 and with different amounts of kindlin-3-His_6 (Fig. 3*D*, *bottom panel*). The rationale is that if integrin β2 tail, kindlin-3, and RACK1 form a ternary complex, a ternary complex could still be formed using the integrin β2 N741A mutant because RACK1 could serve as the bridging molecule for kindlin-3 and integrin β2 N741A despite the fact that kindlin-3 interacts poorly with β2 N741A (Fig. 3*D*, *schematic*). Indeed, kindlin-3-His_6 was detected in both samples of β2 tail peptides in the presence of His_6-RACK1, and the levels of association were comparable. These data suggest that the integrin β2 tail, kindlin-3, and RACK1 can form a ternary complex.

Interaction and Co-localization Analyses of Kindlin-3 and RACK1 in T Cells—To examine the interaction of endogenous kindlin-3 and RACK1, we performed co-immunoprecipitation assays. SKW3 human T lymphoma cells (53) were seeded into microtiter wells with or without ICAM-1-coating in the presence of SDF-1α. After incubation for 30 min under culture conditions, cells were collected and lysed, and immunoprecipitation of kindlin-3 was performed using a kindlin-3-specific mAb (clone 9) that we generated. Immunoblotting of precipitated kindlin-3 was performed using another kindlin-3-specific mAb (clone 229A). High levels of RACK1 co-precipitated with kindlin-3 from lysates of SDF-1α-treated cells that were allowed to adhere to immobilized ICAM-1 compared with cells treated with SDF-1α alone (Fig. 4). Integrin αLβ2 also co-precipitated with kindlin-3 in both samples.

To further investigate the physiological relevance of kindlin-3 and RACK1 interaction, we performed immunofluorescence microscopy of T cells. First we examined the localization of these molecules in SDF-1α-treated SKW3 cells that adhered and spread on immobilized ICAM-1. Actin and nuclei staining were also performed. MyH9 staining was also included because it has been reported to localize at the rear of migrating T cells (54). Kindlin-3 and RACK1 localized at the migrating front, whereas MyH9 localized predominantly at the rear of the cells (Fig. 5). Representative intensity plots showing the distributions of these molecules in the polarized cells are shown (Fig. 5, *insets*). In double-staining experiments, kindlin-3 and RACK1 were observed to co-localize at the front of the polarized cells (Fig. 6). Similar observations were made in human T lymphoblasts that adhered and spread on immobilized ICAM-1 in the presence of SDF-1α (Fig. 7). To rule out interdonor variation, we also performed additional double-staining experiments with T lymphoblasts from two other donors (supplemental Fig. S3 and Fig. S4), and the results were consistent.

DISCUSSION

Kindlins are widely reported as co-activators with talin of integrins, but there is gaining evidence that suggests kindlins as mediators of integrin outside-in signaling. In human keratinocytes, kindlin-1 has been reported to form a complex with the β1 integrin, α-actinin, migfilin, and FAK, and it controls lamellipodia formation via RhoGTPase signaling pathways (55). In mouse osteoblastic cells, kindlin-2 knockdown abrogated β1 integrin-mediated activation of Rac1, Akt, and AP-1, which are involved in cell adhesion and proliferation (56). Platelets from mice deficient in kindlin-3 adhered to fibrinogen in the presence of Mn^{2+} that activates integrin αIIbβ3, but they failed to form lamellipodia (15). Here we show that kindlin-3 is required for outside-in signaling of integrins αLβ2 and αIIbβ3. The requirement for kindlin-3 in inside-out activation of these integrins was bypassed using mutants αLβ2N329S and αIIbβ3N339S, which are constitutively activated with high ligand binding affinity (45). Electric cell-substrate impedance-sensing measurements showed that cells with reduced kindlin-3 expression were defective in spreading despite expressing these activated mutants. Whether kindlin-3, like its paralogs kindlin-1 and kindlin-2, is involved in mediating integrin αLβ2- or αIIbβ3-derived RhoGTPase signaling remains to be determined.

A recent study of integrin αLβ2-mediated T lymphocyte adhesion on ICAM-1 has shown that kindlin-3 is required for T cell receptor-induced clustering of activated αLβ2 in early focal assemblies rather than inducing integrin αLβ2 conformational changes (44). Our data suggest that kindlin-3 may induce micro-clustering of integrin αLβ2. The overall strength of cell adhesion or avidity is dependent on the affinity of individual adhesion molecule to its ligand and the number of adhesion molecules at the contact site (57). Clustering of integrins, therefore, plays an important role in adhesion strengthening. At present, we do not have information on the mechanism by which kindlin-3 induces integrin αLβ2 micro-clustering. Its PH domain may have an important role in this process by binding to membrane phosphatidylinositol phosphate(s). It is interesting to note that talin, which is a well established direct activator of integrins, can induce β3 integrin clustering, and this is dependent on its F2 subdomain binding to phosphatidylinositol 4,5-diphosphate (58, 59).

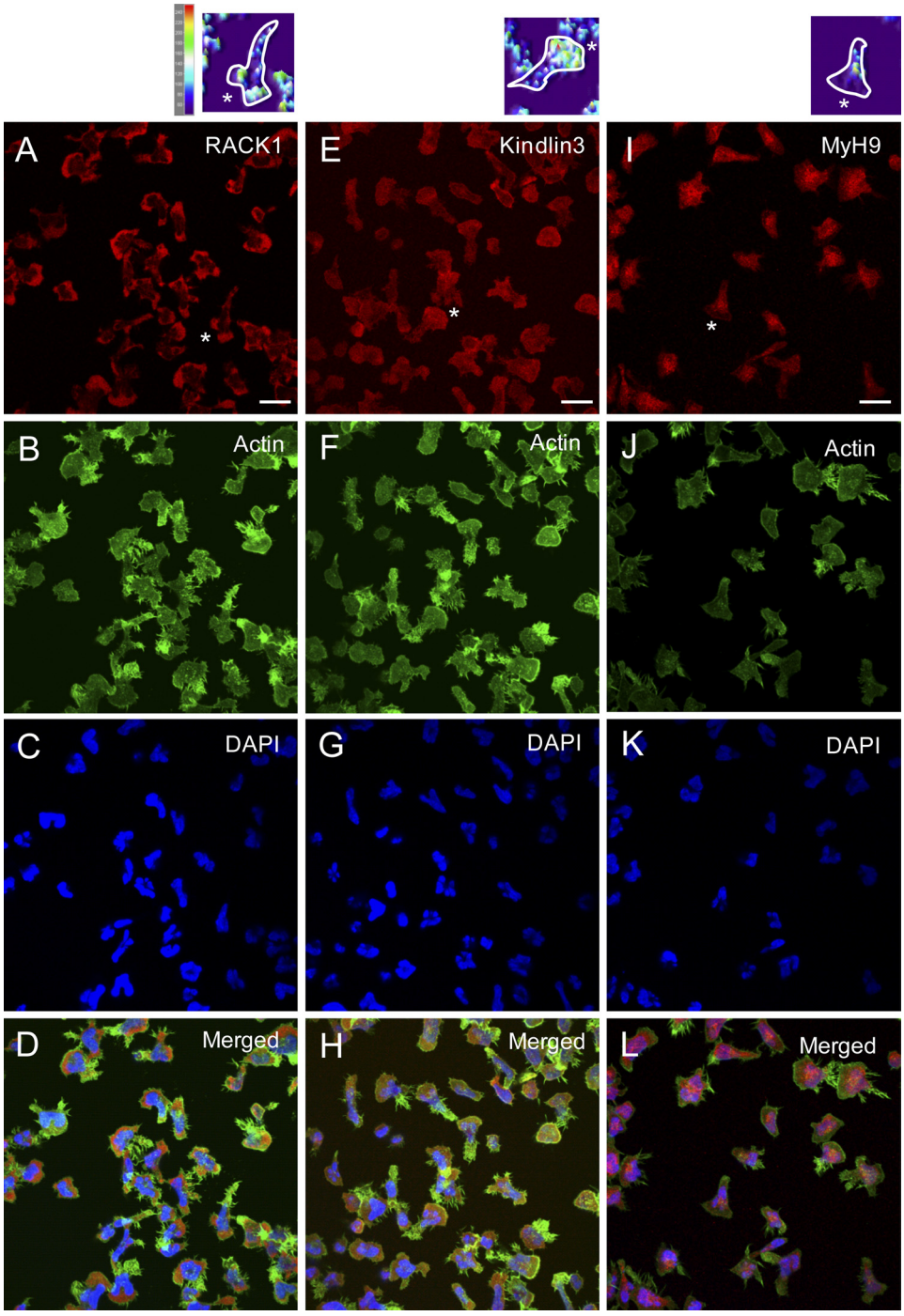


FIGURE 5. **Immunofluorescence staining of endogenous kindlin-3, RACK1, and MyH9 in SKW3 cells.** Cells that adhered to ICAM-1-coated coverslips in the presence of SDF-1α were fixed, permeabilized, and stained for kindlin-3, RACK1, and MyH9 with relevant antibodies as described under "Experimental Procedures." F-actin and nucleus were stained with Alexa Fluor® 488-conjugated phalloidin and DAPI, respectively. *Scale bar*, 20 μm. Intensity plots of representative cells are shown (*top panel*).

Aforementioned, we and others have shown that kindlins are involved in integrin outside-in signaling. We have provided evidence that kindlin-3 interacts with RACK1, a seven-blade propeller WD-repeat scaffold protein (32). Two other RACKs are the PKCϵ and PKCα receptors β′-COP and Pick1, respectively (60, 61). RACK1 binds many cytoplasmic molecules (32), and the interactions involved different blades of RACK1. For example, c-Src (blade 6), RAID (blades 5, 6, and 7), PKCβII (blades 3 and 6), and integrin β1, β2, and β5 tails (blades 5, 6, and 7) (32, 33). RACK1 stabilizes the activated forms of PKCs and enables the translocation of PKCs to different sites in the cell (62), but it inhibits the activity of Src family kinases (63). However, it has been reported that RACK1 does not interact with PKCβ or Src in chemokine-treated Jurkat T cells (47). There are also conflicting reports as to whether RACK1 inhibits or promotes cell migration. RACK1 overexpression in integrin αIIbβ3-expressing CHO cells increased the number of actin stress fibers and focal contacts, and the cells showed impeded migration on

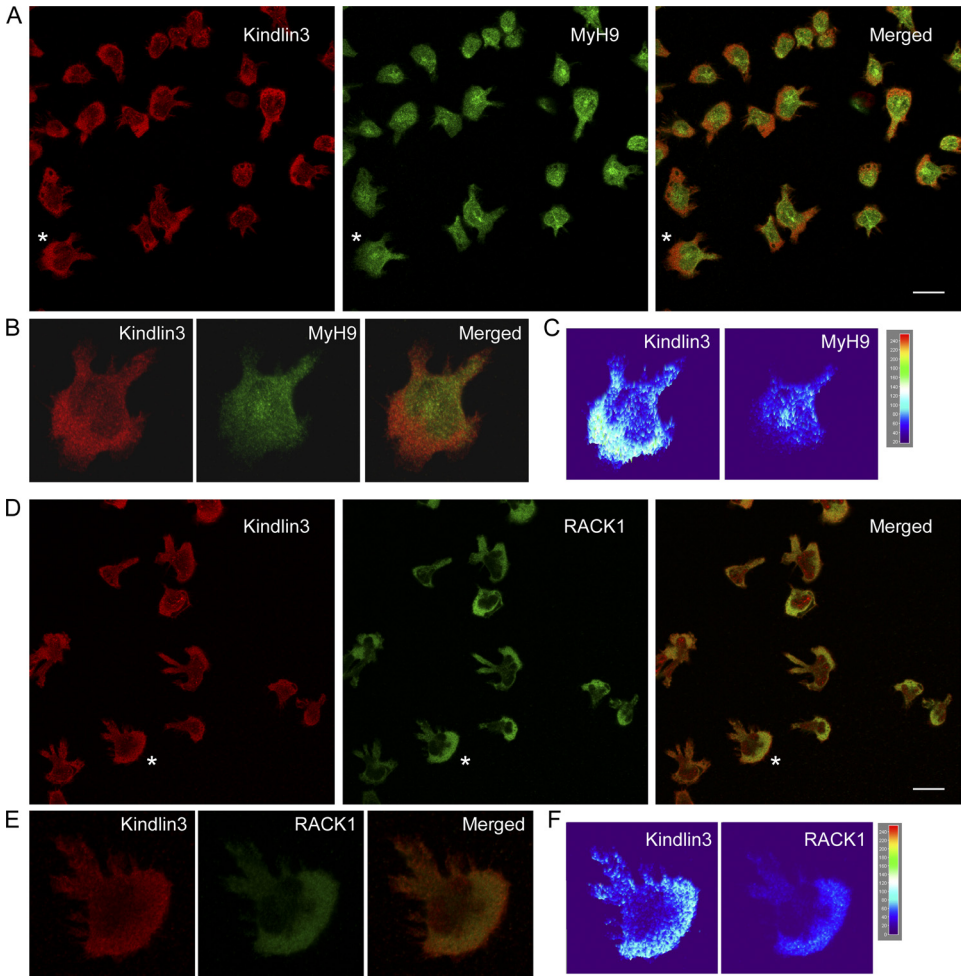


FIGURE 6. **Co-localization of endogenous kindlin-3 and RACK1 in SKW3 cells.** Shown is immunofluorescence staining of kindlin-3 and MyH9 (*A*) or kindlin-3 and RACK1 (*D*) in SKW3 cells that adhered to ICAM-1-coated coverslips in the presence of SDF-1α. *B* and *E*, shown are magnified images of selected cells (*). *C* and *F*, intensity plots of selected cells (*). *Scale bar*, 20 μm.

fibrinogen (34). RACK1 has been shown to localize to nascent focal complexes and regulates cell protrusion (64). It has also been reported that RACK1 localized to the leading edge of chemoattractant-induced polarized Jurkat and HL-60 cells, but it associates with Gβγ and act as a negative regulator of directed cell migration (47). Thus, the functions of RACK1 may be cell type-specific and dependent on the type of cellular stimuli. Adding to the complexity is that RACK1 has been reported as a eukaryotic ribosomal protein (65).

The functional consequences of kindlin-3-RACK1 interaction remain to be delineated. Our data suggest that the interaction is dependent on kindlin-3 PH domain and RACK1 blades 5–7. We cannot exclude other blades of RACK1 that may be important for this interaction because we were unable to obtain soluble expression of other RACK1 blades using the *E. coli*. expression system. Because blades 5–7 of RACK1 contain binding sites for other proteins, it remains to be determined if RACK1 can interact with these molecules simultaneously or with selective molecule(s) at any one time. RACK1 has been shown to bind the membrane proximal sequence Lys-702–Leu-721 of the integrin β2 tail (33), whereas kindlin-3 binds to the C-terminal sequence of the integrin β2 tail that contains the ^{741}NPKF motif (14). Our data suggest that the integrin β2 tail, kindlin-3, and RACK1 can form a ternary complex. It is possible that RACK1 recruits kinases such as c-Src or activated PKCs to this complex, which may modulate both ligand binding and signaling activity of the β2 integrins.

The kindlin-3 and talin binding sites in the integrin β2 tail are in close proximity of each other. Whether kindlin-3 and talin binding to the integrin β2 tail influence the interaction of kindlin-3 with RACK1 remains to be determined. Currently it has yet to be shown that kindlin-3 and talin head domain can bind a single integrin β2 tail simultaneously. There is little evidence suggesting direct interaction between kindlins and talin head domain. The fate of these two molecules after integrin ligand binding is also unclear. Several models of integrin activation by kindlins and talin have been proposed (25). The *cis* cooperation model positions kindlin and talin on a single integrin tail, but the sequential binding and *trans* co-operation models do not. Our findings using the SKW3 T cells suggest that integrin αLβ2 engagement by ICAM-1 promotes kindlin-3 and RACK1 interaction. This may be important in integrin αLβ2 outside-in signaling. More studies are needed to delineate the function of kindlin-3-RACK1 interaction in this context.

Unlike kindlin-3, which interacts with RACK1, we were unable to detect significant interaction between kindlin-2 and

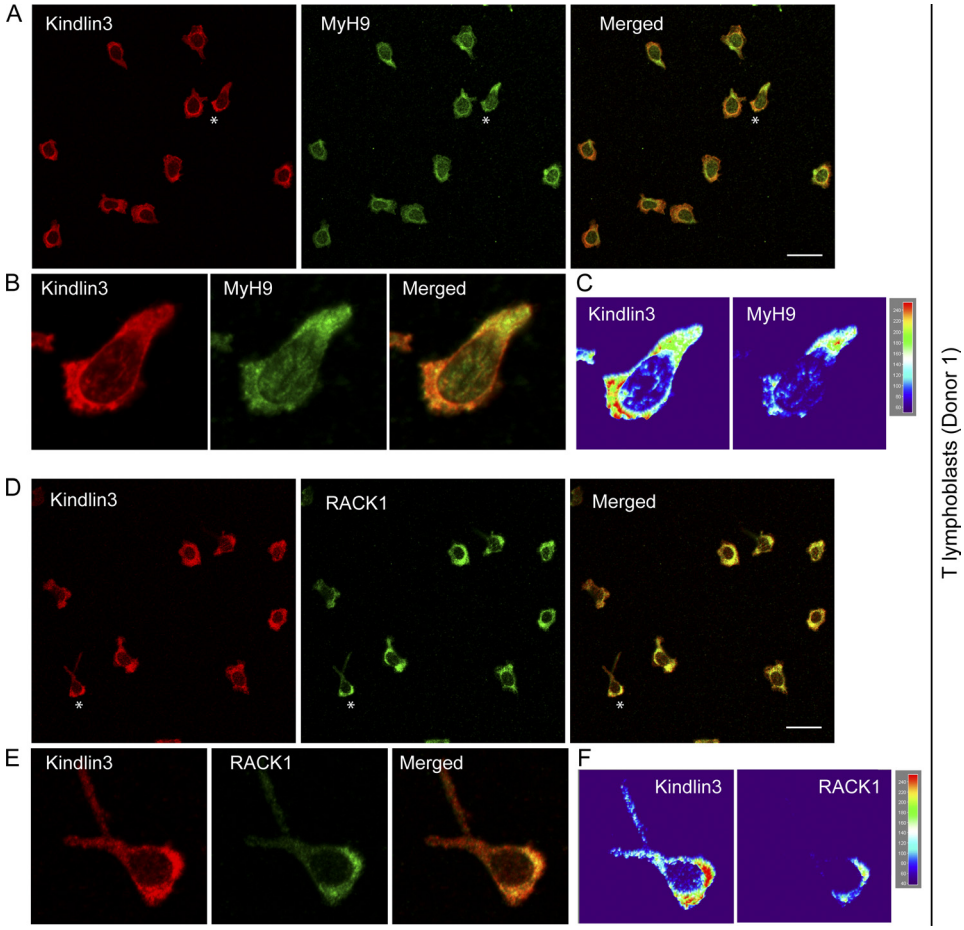


FIGURE 7. **Co-localization of endogenous kindlin-3 and RACK1 in human T lymphoblasts.** Shown is immunofluorescence staining of kindlin-3 and MyH9 (*A*) or kindlin-3 and RACK1 (*D*) in human T lymphoblasts that adhered to ICAM-1-coated coverslips in the presence of SDF-1α. *B* and *E*, shown are magnified images of selected cells (*). *C* and *F*, shown are intensity plots of selected cells (*). *Scale bar*, 20 μm. Cells were from one donor.

RACK1. RACK1 has been reported to be important for nascent focal adhesion complex formation (64, 66), and its localization was detected in preforming adhesion sites known as spreading initiation centers but not in mature focal adhesion sites (67). Kindlin-3 has been shown to localize at the spreading lamellipodia, but not focal adhesion sites, of human umbilical vein endothelial cells cultured on integrin substrates, whereas kindlin-2 was detected in both cellular structures (26). Indeed, we observed that kindlin-3 and RACK1 localized at the migrating front of polarized T cells. By contrast, MyH9 was localized at the rear of these cells, which is in line with previous observations (54). We did not perform kindlin-2 staining of these cells because we could not detect kindlin-2 protein in these cells by immunoblotting (data not shown), although kindlin-2 mRNA expression was detected at a low level in lymphoid organs (68). The physiological significance of kindlin-3, but not kindlin-2, interacting with RACK1 remains to be determined. It may be interesting to examine this in human umbilical vein endothelial cells that express both kindlins (26).

In summary, we have shown that kindlin-3 plays a role in integrin αLβ2 outside-in signaling, and it can induce integrin αLβ2 micro-clustering. We also report that kindlin-3 interacts with the scaffold protein RACK1 that is known to have multiple cytoplasmic binding partners. The biological importance of this interaction in integrin outside-in signaling awaits further studies.

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REFERENCES

1. Hynes, R. O. (2002) Integrins. Bidirectional, allosteric signaling machines. *Cell* **110,** 673–687
2. Streuli, C. H. (2009) Integrins and cell-fate determination. *J. Cell Sci.* **122,** 171–177
3. Shattil, S. J., Kim, C., and Ginsberg, M. H. (2010) The final steps of integrin activation. The end game. *Nat. Rev. Mol. Cell Biol.* **11,** 288–300
4. Abram, C. L., and Lowell, C. A. (2009) The ins and outs of leukocyte integrin signaling. *Annu. Rev. Immunol.* **27,** 339–362
5. Calderwood, D. A., Shattil, S. J., and Ginsberg, M. H. (2000) Integrins and actin filaments. Reciprocal regulation of cell adhesion and signaling. *J. Biol. Chem.* **275,** 22607–22610
6. Critchley, D. R., and Gingras, A. R. (2008) Talin at a glance. *J. Cell Sci.* **121,** 1345–1347
7. Calderwood, D. A., Zent, R., Grant, R., Rees, D. J., Hynes, R. O., and Ginsberg, M. H. (1999) The Talin head domain binds to integrin β subunit

cytoplasmic tails and regulates integrin activation. *J. Biol. Chem.* **274,** 28071–28074
8. Tadokoro, S., Shattil, S. J., Eto, K., Tai, V., Liddington, R. C., de Pereda, J. M., Ginsberg, M. H., and Calderwood, D. A. (2003) Talin binding to integrin β tails. A final common step in integrin activation. *Science* **302,** 103–106
9. Kim, M., Carman, C. V., and Springer, T. A. (2003) Bidirectional transmembrane signaling by cytoplasmic domain separation in integrins. *Science* **301,** 1720–1725
10. Anthis, N. J., Wegener, K. L., Ye, F., Kim, C., Goult, B. T., Lowe, E. D., Vakonakis, I., Bate, N., Critchley, D. R., Ginsberg, M. H., and Campbell, I. D. (2009) The structure of an integrin/talin complex reveals the basis of inside-out signal transduction. *EMBO J.* **28,** 3623–3632
11. Luo, B. H., Carman, C. V., and Springer, T. A. (2007) Structural basis of integrin regulation and signaling. *Annu. Rev. Immunol.* **25,** 619–647
12. Arnaout, M. A., Goodman, S. L., and Xiong, J. P. (2007) Structure and mechanics of integrin-based cell adhesion. *Curr. Opin. Cell Biol.* **19,** 495–507
13. Harburger, D. S., Bouaouina, M., and Calderwood, D. A. (2009) Kindlin-1 and -2 directly bind the C-terminal region of β integrin cytoplasmic tails and exert integrin-specific activation effects. *J. Biol. Chem.* **284,** 11485–11497
14. Moser, M., Bauer, M., Schmid, S., Ruppert, R., Schmidt, S., Sixt, M., Wang, H. V., Sperandio, M., and Fässler, R. (2009) Kindlin-3 is required for β2 integrin-mediated leukocyte adhesion to endothelial cells. *Nat. Med.* **15,** 300–305
15. Moser, M., Nieswandt, B., Ussar, S., Pozgajova, M., and Fässler, R. (2008) Kindlin-3 is essential for integrin activation and platelet aggregation. *Nat. Med.* **14,** 325–330
16. Ma, Y. Q., Qin, J., Wu, C., and Plow, E. F. (2008) Kindlin-2 (Mig-2). A co-activator of β3 integrins. *J. Cell Biol.* **181,** 439–446
17. Meves, A., Stremmel, C., Gottschalk, K., and Fässler, R. (2009) The Kindlin protein family. New members to the club of focal adhesion proteins. *Trends Cell Biol.* **19,** 504–513
18. Goult, B. T., Bouaouina, M., Harburger, D. S., Bate, N., Patel, B., Anthis, N. J., Campbell, I. D., Calderwood, D. A., Barsukov, I. L., Roberts, G. C., and Critchley, D. R. (2009) The structure of the N-terminus of kindlin-1. A domain important for αiibβ3 integrin activation. *J. Mol. Biol.* **394,** 944–956
19. Qu, H., Tu, Y., Shi, X., Larjava, H., Saleem, M. A., Shattil, S. J., Fukuda, K., Qin, J., Kretzler, M., and Wu, C. (2011) Kindlin-2 regulates podocyte adhesion and fibronectin matrix deposition through interactions with phosphoinositides and integrins. *J. Cell Sci.* **124,** 879–891
20. Montanez, E., Ussar, S., Schifferer, M., Bösl, M., Zent, R., Moser, M., and Fässler, R. (2008) Kindlin-2 controls bidirectional signaling of integrins. *Genes Dev.* **22,** 1325–1330
21. Tu, Y., Wu, S., Shi, X., Chen, K., and Wu, C. (2003) Migfilin and Mig-2 link focal adhesions to filamin and the actin cytoskeleton and function in cell shape modulation. *Cell* **113,** 37–47
22. Ithychanda, S. S., Das, M., Ma, Y. Q., Ding, K., Wang, X., Gupta, S., Wu, C., Plow, E. F., and Qin, J. (2009) Migfilin, a molecular switch in regulation of integrin activation. *J. Biol. Chem.* **284,** 4713–4722
23. Das, M., Ithychanda, S. S., Qin, J., and Plow, E. F. (2011) Migfilin and filamin as regulators of integrin activation in endothelial cells and neutrophils. *PLoS One* **6,** e26355
24. Kiema, T., Lad, Y., Jiang, P., Oxley, C. L., Baldassarre, M., Wegener, K. L., Campbell, I. D., Ylänne, J., and Calderwood, D. A. (2006) The molecular basis of filamin binding to integrins and competition with talin. *Mol. Cell* **21,** 337–347
25. Moser, M., Legate, K. R., Zent, R., and Fässler, R. (2009) The tail of integrins, talin, and kindlins. *Science* **324,** 895–899
26. Bialkowska, K., Ma, Y. Q., Bledzka, K., Sossey-Alaoui, K., Izem, L., Zhang, X., Malinin, N., Qin, J., Byzova, T., and Plow, E. F. (2010) The integrin co-activator Kindlin-3 is expressed and functional in a non-hematopoietic cell, the endothelial cell. *J. Biol. Chem.* **285,** 18640–18649
27. Kuijpers, T. W., van de Vijver, E., Weterman, M. A., de Boer, M., Tool, A. T., van den Berg, T. K., Moser, M., Jakobs, M. E., Seeger, K., Sanal, O., Unal, S., Cetin, M., Roos, D., Verhoeven, A. J., and Baas, F. (2009) LAD-1/variant syndrome is caused by mutations in FERMT3. *Blood* **113,** 4740–4746
28. Svensson, L., Howarth, K., McDowall, A., Patzak, I., Evans, R., Ussar, S., Moser, M., Metin, A., Fried, M., Tomlinson, I., and Hogg, N. (2009) Leukocyte adhesion deficiency-III is caused by mutations in KINDLIN3 affecting integrin activation. *Nat. Med.* **15,** 306–312
29. Malinin, N. L., Zhang, L., Choi, J., Ciocea, A., Razorenova, O., Ma, Y. Q., Podrez, E. A., Tosi, M., Lennon, D. P., Caplan, A. I., Shurin, S. B., Plow, E. F., and Byzova, T. V. (2009) A point mutation in KINDLIN3 ablates activation of three integrin subfamilies in humans. *Nat. Med.* **15,** 313–318
30. McDowall, A., Svensson, L., Stanley, P., Patzak, I., Chakravarty, P., Howarth, K., Sabnis, H., Briones, M., and Hogg, N. (2010) Two mutations in the KINDLIN3 gene of a new leukocyte adhesion deficiency III patient reveal distinct effects on leukocyte function *in vitro*. *Blood* **115,** 4834–4842
31. Krüger, M., Moser, M., Ussar, S., Thievessen, I., Luber, C. A., Forner, F., Schmidt, S., Zanivan, S., Fässler, R., and Mann, M. (2008) SILAC mouse for quantitative proteomics uncovers kindlin-3 as an essential factor for red blood cell function. *Cell* **134,** 353–364
32. McCahill, A., Warwicker, J., Bolger, G. B., Houslay, M. D., and Yarwood, S. J. (2002) The RACK1 scaffold protein. A dynamic cog in cell response mechanisms. *Mol. Pharmacol.* **62,** 1261–1273
33. Liliental, J., and Chang, D. D. (1998) Rack1, a receptor for activated protein kinase C, interacts with integrin β subunit. *J. Biol. Chem.* **273,** 2379–2383
34. Buensuceso, C. S., Woodside, D., Huff, J. L., Plopper, G. E., and O'Toole, T. E. (2001) The WD protein Rack1 mediates protein kinase C and integrin-dependent cell migration. *J. Cell Sci.* **114,** 1691–1698
35. Buensuceso, C. S., Obergfell, A., Soriani, A., Eto, K., Kiosses, W. B., Arias-Salgado, E. G., Kawakami, T., and Shattil, S. J. (2005) Regulation of outside-in signaling in platelets by integrin-associated protein kinase C β. *J. Biol. Chem.* **280,** 644–653
36. Hildreth, J. E., Gotch, F. M., Hildreth, P. D., and McMichael, A. J. (1983) A human lymphocyte-associated antigen involved in cell-mediated lympholysis. *Eur. J. Immunol.* **13,** 202–208
37. Coller, B. S., Peerschke, E. I., Scudder, L. E., and Sullivan, C. A. (1983) A murine monoclonal antibody that completely blocks the binding of fibrinogen to platelets produces a thrombasthenic-like state in normal platelets and binds to glycoproteins IIb and/or IIIa. *J. Clin. Invest.* **72,** 325–338
38. Luo, B. H., Takagi, J., and Springer, T. A. (2004) Locking the β3 integrin I-like domain into high and low affinity conformations with disulfides. *J. Biol. Chem.* **279,** 10215–10221
39. Tan, S. M., Walters, S. E., Mathew, E. C., Robinson, M. K., Drbal, K., Shaw, J. M., and Law, S. K. (2001) Defining the repeating elements in the cysteine-rich region (CRR) of the CD18 integrin β 2 subunit. *FEBS Lett.* **505,** 27–30
40. Rajamanonmani, R., Nkenfou, C., Clancy, P., Yau, Y. H., Shochat, S. G., Sukupolvi-Petty, S., Schul, W., Diamond, M. S., Vasudevan, S. G., and Lescar, J. (2009) On a mouse monoclonal antibody that neutralizes all four dengue virus serotypes. *J. Gen. Virol.* **90,** 799–809
41. Myszka, D. G. (1999) Improving biosensor analysis. *J. Mol. Recognit.* **12,** 279–284
42. Vararattanavech, A., Tang, M. L., Li, H. Y., Wong, C. H., Law, S. K., Torres, J., and Tan, S. M. (2008) Permissive transmembrane helix heterodimerization is required for the expression of a functional integrin. *Biochem. J.* **410,** 495–502
43. Vararattanavech, A., Lin, X., Torres, J., and Tan, S. M. (2009) Disruption of the integrin αLβ2 transmembrane domain interface by β2 Thr-686 mutation activates αLβ2 and promotes micro-clustering of the αL subunits. *J. Biol. Chem.* **284,** 3239–3249
44. Feigelson, S. W., Grabovsky, V., Manevich-Mendelson, E., Pasvolsky, R., Shulman, Z., Shinder, V., Klein, E., Etzioni, A., Aker, M., and Alon, R. (2011) Kindlin-3 is required for the stabilization of TCR-stimulated LFA-1:ICAM-1 bonds critical for lymphocyte arrest and spreading on dendritic cells. *Blood* **117,** 7042–7052
45. Cheng, M., Foo, S. Y., Shi, M. L., Tang, R. H., Kong, L. S., Law, S. K., and Tan, S. M. (2007) Mutation of a conserved asparagine in the I-like domain promotes constitutively active integrins αLβ2 and αIIbβ3. *J. Biol. Chem.* **282,** 18225–18232
46. Giaever, I., and Keese, C. R. (1993) A morphological biosensor for mam-

malian cells. *Nature* **366,** 591–592
47. Chen, S., Lin, F., Shin, M. E., Wang, F., Shen, L., and Hamm, H. E. (2008) RACK1 regulates directional cell migration by acting on Gβγ at the interface with its effectors PLC β and PI3Kγ. *Mol. Biol. Cell* **19,** 3909–3922
48. Rodriguez, M. M., Ron, D., Touhara, K., Chen, C. H., and Mochly-Rosen, D. (1999) RACK1, a protein kinase C anchoring protein, coordinates the binding of activated protein kinase C and select pleckstrin homology domains *in vitro*. *Biochemistry* **38,** 13787–13794
49. Pluskota, E., Dowling, J. J., Gordon, N., Golden, J. A., Szpak, D., West, X. Z., Nestor, C., Ma, Y. Q., Bialkowska, K., Byzova, T., and Plow, E. F. (2011) The integrin coactivator kindlin-2 plays a critical role in angiogenesis in mice and zebrafish. *Blood* **117,** 4978–4987
50. Shen, Z., Ye, Y., Dong, L., Vainionpää, S., Mustonen, H., Puolakkainen, P., and Wang, S. (2012) Kindlin-2. A novel adhesion protein related to tumor invasion, lymph node metastasis, and patient outcome in gastric cancer. *Am. J. Surg.* **203,** 222–229
51. He, Y., Esser, P., Schacht, V., Bruckner-Tuderman, L., and Has, C. (2011) Role of kindlin-2 in fibroblast functions: implications for wound healing. *J. Invest. Dermatol.* **131,** 245–256
52. Manevich-Mendelson, E., Feigelson, S. W., Pasvolsky, R., Aker, M., Grabovsky, V., Shulman, Z., Kilic, S. S., Rosenthal-Allieri, M. A., Ben-Dor, S., Mory, A., Bernard, A., Moser, M., Etzioni, A., and Alon, R. (2009) Loss of Kindlin-3 in LAD-III eliminates LFA-1 but not VLA-4 adhesiveness developed under shear flow conditions. *Blood* **114,** 2344–2353
53. Dustin, M. L., Rothlein, R., Bhan, A. K., Dinarello, C. A., and Springer, T. A. (1986) Induction by IL 1 and interferon-γ. Tissue distribution, biochemistry, and function of a natural adherence molecule (ICAM-1). *J. Immunol.* **137,** 245–254
54. Morin, N. A., Oakes, P. W., Hyun, Y. M., Lee, D., Chin, Y. E., Chin, E. Y., King, M. R., Springer, T. A., Shimaoka, M., Tang, J. X., Reichner, J. S., and Kim, M. (2008) Nonmuscle myosin heavy chain IIA mediates integrin LFA-1 de-adhesion during T lymphocyte migration. *J. Exp. Med.* **205,** 195–205
55. Has, C., Herz, C., Zimina, E., Qu, H. Y., He, Y., Zhang, Z. G., Wen, T. T., Gache, Y., Aumailley, M., and Bruckner-Tuderman, L. (2009) Kindlin-1 Is required for RhoGTPase-mediated lamellipodia formation in keratinocytes. *Am. J. Pathol.* **175,** 1442–1452
56. Jung, G. Y., Park, Y. J., and Han, J. S. (2011) Mediation of Rac1 activation by kindlin-2, an essential function in osteoblast adhesion, spreading, and proliferation. *J. Cell. Biochem.* **112,** 2541–2548
57. Carman, C. V., and Springer, T. A. (2003) Integrin avidity regulation. Are changes in affinity and conformation underemphasized? *Curr. Opin. Cell Biol.* **15,** 547–556
58. Cluzel, C., Saltel, F., Lussi, J., Paulhe, F., Imhof, B. A., and Wehrle-Haller, B. (2005) The mechanisms and dynamics of αvβ3 integrin clustering in living cells. *J. Cell Biol.* **171,** 383–392
59. Saltel, F., Mortier, E., Hytönen, V. P., Jacquier, M. C., Zimmermann, P., Vogel, V., Liu, W., and Wehrle-Haller, B. (2009) New PI(4,5)P2- and membrane proximal integrin-binding motifs in the talin head control β3-integrin clustering. *J. Cell Biol.* **187,** 715–731
60. Csukai, M., Chen, C. H., De Matteis, M. A., and Mochly-Rosen, D. (1997) The coatomer protein β′-COP, a selective binding protein (RACK) for protein kinase Cε. *J. Biol. Chem.* **272,** 29200–29206
61. Staudinger, J., Zhou, J., Burgess, R., Elledge, S. J., and Olson, E. N. (1995) PICK1. A perinuclear binding protein and substrate for protein kinase C isolated by the yeast two-hybrid system. *J. Cell Biol.* **128,** 263–271
62. Mochly-Rosen, D., and Gordon, A. S. (1998) Anchoring proteins for protein kinase C. A means for isozyme selectivity. *FASEB J.* **12,** 35–42
63. Chang, B. Y., Conroy, K. B., Machleder, E. M., and Cartwright, C. A. (1998) RACK1, a receptor for activated C kinase and a homolog of the β subunit of G proteins, inhibits activity of src tyrosine kinases and growth of NIH 3T3 cells. *Mol. Cell. Biol.* **18,** 3245–3256
64. Cox, E. A., Bennin, D., Doan, A. T., O'Toole, T., and Huttenlocher, A. (2003) RACK1 regulates integrin-mediated adhesion, protrusion, and chemotactic cell migration via its Src-binding site. *Mol. Biol. Cell* **14,** 658–669
65. Rabl, J., Leibundgut, M., Ataide, S. F., Haag, A., and Ban, N. (2011) Crystal structure of the eukaryotic 40 S ribosomal subunit in complex with initiation factor 1. *Science* **331,** 730–736
66. Doan, A. T., and Huttenlocher, A. (2007) RACK1 regulates Src activity and modulates paxillin dynamics during cell migration. *Exp. Cell Res.* **313,** 2667–2679
67. de Hoog, C. L., Foster, L. J., and Mann, M. (2004) RNA and RNA binding proteins participate in early stages of cell spreading through spreading initiation centers. *Cell* **117,** 649–662
68. Ussar, S., Wang, H. V., Linder, S., Fässler, R., and Moser, M. (2006) The Kindlins. Subcellular localization and expression during murine development. *Exp. Cell Res.* **312,** 3142–3151